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14. ABSTRACT

The Epithelial-to-Mesenchymal Transition (EMT) is a conserved developmental process that is thought to be reactivated during the metastasis of epithelial cancers such as breast cancer. This study seeks to identify genes commonly regulated in the EMT, and identify key regulators of the process. An EMT core gene signature of approximately 1000 genes was generated, from which the transcription factor Zeb1 emerged as a potent key regulator of the EMT. Zeb1 demonstrated a much stronger EMT-inducing ability than previously known factors, and was demonstrated to be both sufficient for EMT and necessary for maintenance of the mesenchymal phenotype. Efforts are underway to construct the genetic circuit stemming from Zeb1. A Zeb1-induced EMT model is also being employed to study the role of EMT in cancer metastasis in vivo.

15. SUBJECT TERMS
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Introduction

Breast cancer is the most common form of cancer in women in the United States (*1*). Mortality results not from the primary tumor, but from complications arising from metastases that have spread beyond the breast itself. Hence, the study of breast cancer progression and metastasis is as important as the study of what causes the cancer to arise in the first place. The Epithelial-to-Mesenchymal Transition (EMT) is a conserved embryonic developmental process where epithelial germ layer cells acquire the properties of mesenchymal cells, allowing the cells to migrate away from their original site (*2, 3*). This process is thought to be reactivated during cancer metastasis, and may facilitate the dissemination of cancer cells to distant organs. The EMT is not well-defined at a molecular level, and it is not known which aspects of the EMT may be important in cancer metastasis. This study seeks to identify genes that behave consistently upon EMT, regardless of the initial triggering event. That information will be used to identify candidate regulators of the EMT. Successful candidates will serve as anchor points to molecularly define the core genetic circuit that controls the EMT. The eventual hope, beyond the scope of this study, is to use this information to guide the development of measures that can inhibit the process of EMT, and along with it, slow or stop metastatic progression.

Body

1. Generate a core gene expression signature of the EMT using the HMLE-derived cell lines

Cell lines existing in the lab were used to generate microarray gene expression profiles. The human mammary epithelial line HMLE was used, along with derivative mesenchymal lines constitutively expressing Snail, Twist, Goosecoid, TGF-β1 and short hairpin RNA against E-cadherin. The raw data from the microarray experiment was filtered by p-value with an individual test cutoff of 0.05, with Bonferroni correction (*4*). The result was a statistically significant core EMT signature of approximately 1000 genes which are consistently up- or down-regulated in all five mesenchymal lines compared with their epithelial controls (Figure 1).

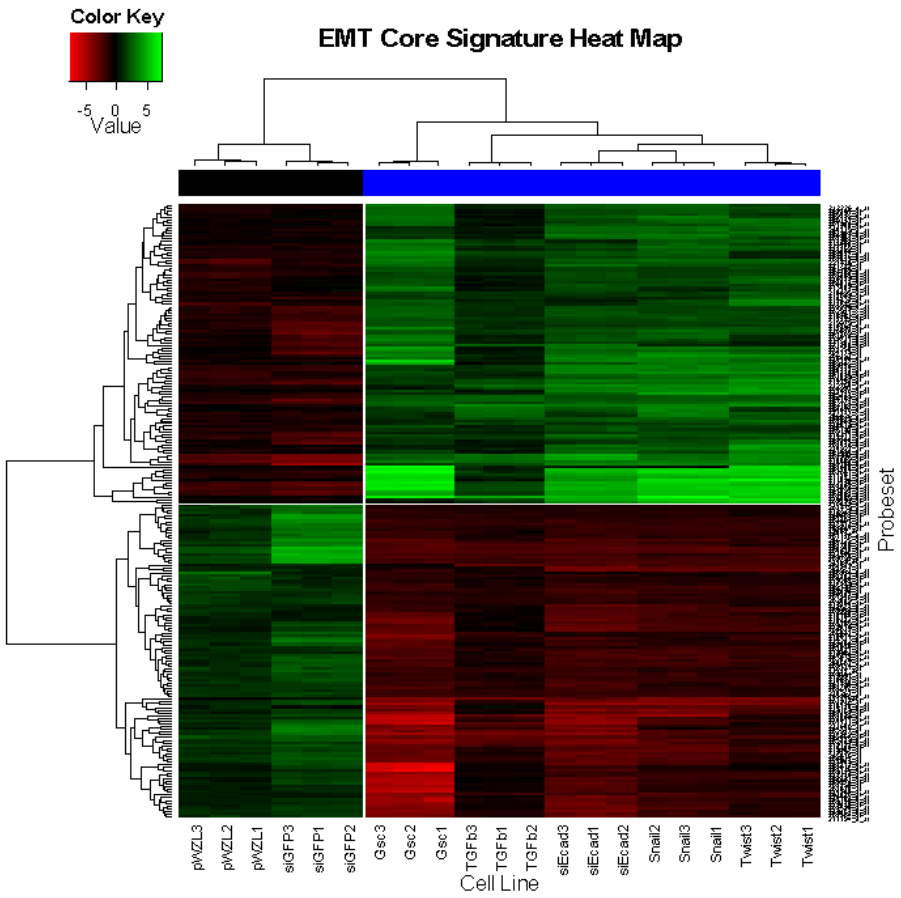

Figure 1 EMT Core Signature showing a substantial overlap between mesenchymal cells derived from various treatments.

2. Use the core gene signature to find key components of the core regulatory circuitry

Attempts to draw a gene network solely from the microarray data were unsuccessful. Several common bioinformatics procedures were attempted and did not yield strong candidates (*5, 6*). Neither did correlating gene expression to genomic locations commonly amplified or deleted in cancers. Comparing existing cancer metastasis gene signatures to the EMT core signature did not yield any overlap over what would have been expected by chance. Finally, a comparison of the core signature to existing high-quality microarray data from clinical samples of melanoma primary tumor versus melanoma metastases (*7*) produced a significant overlap (Figure 2). Further analysis of the promoters of the overlapping set of 155 genes using the online tool rVISTA (*8*) resulted in the identification of evolutionarily conserved binding sites for the transcription factor Zeb1 as being highly enriched. Zeb1 itself was also upregulated in the EMT core signature.

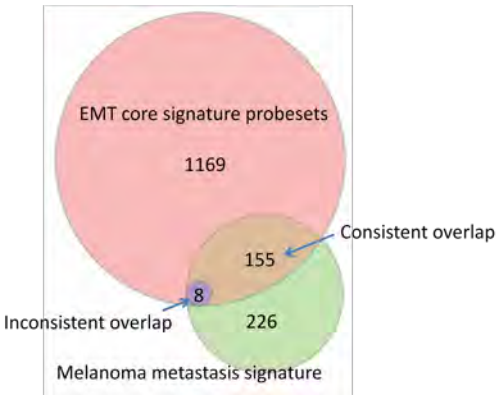

Figure 2 Venn Diagram showing overlap of EMT core signature and a melanoma metastasis signature. Consistent overlap indicates genes upregulated in mesenchymal cells are also upregulated in metastases, and likewise downregulated genes. Inconsistent overlap indicates opposite directional changes. A random comparison of an unrelated gene signature to the EMT core signature would typically give an overlap of 10-20% based on chance, and a consistent:inconsistent ratio of roughly 1:1. This comparison gave an overlap of about 40%, and a ratio of 19:1

3. Test the effect and importance of candidate key compoenents on the EMT in HMLE and other cells *in vitro*

Zeb1 induces a rapid EMT

The ability of Zeb1 to induce an EMT was tested by cloning Zeb1 into a lentiviral vector which drives its constitutive expression in infected cells. It was found that HMLE cells infected with this virus could indeed undergo EMT. Furthermore, it appeared that the EMT occurred on a much shorter timescale than hitherto observed in HMLE cells. Previous work in the lab to induce EMT using Snail and Twist transcription factors, for example, used a different retroviral-based construct(*9*), and expression was driven by a different promoter. Snail and Twist were cloned into the exact same construct as Zeb1 and re-tested on HMLE cells. Once again, they were observed to induce an EMT over the same timescale as the previous retroviral vector, demonstrating that the differing effects of Zeb1 were due to the nature of Zeb1 itself, and not the difference in vector systems. This was the first clue that Zeb1 might be unique among the known EMT inducers.

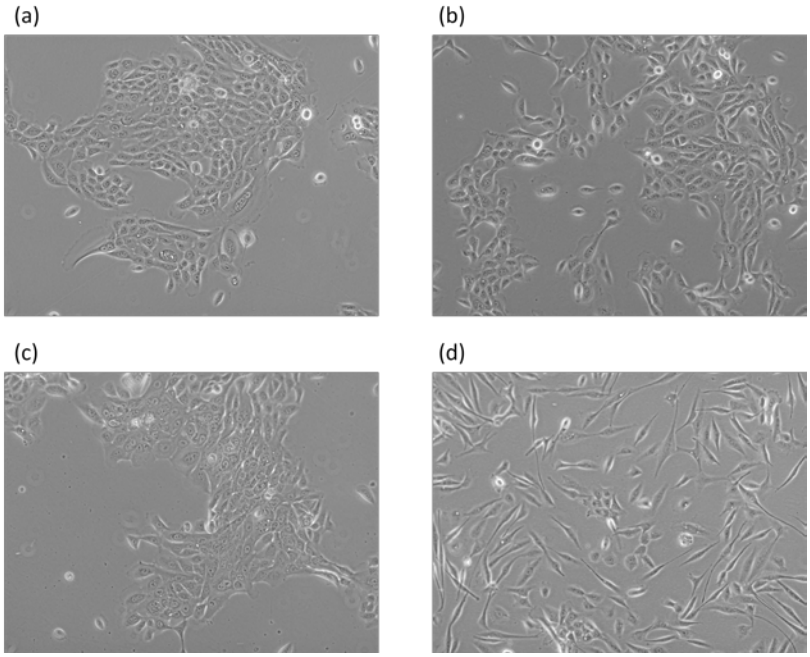

Figure 3 HMLE cells 6 days post-infection with constitutively expressing lentiviruses. (a) GFP-expressing control (b) Snail-expressing (c) Twist-expressing (d) Zeb1-expressing. Zeb1-expressing cells display mesenchymal morphology well before the cells expressing Snail or Twist. The chief visual characteristics are the elongated cells and inability to form epithelial islands of cells.

Zeb1-induced EMT is a true differentiation process

Around this time, the heterogeneity of HMLE cells and indeed other cancer lines was becoming more appreciated. Even though HMLE cells are primarily epithelial, there exists a minor population of cells that are mesenchymal (*10*). These mesenchymal cells grow more slowly and are more resistant to cell death by various means (*11*). It was also observed that HMLE cells infected with the Twist-expressing lentivirus always displayed more cell death than cells infected with Snail-, Zeb1- or GFP-expressing lentiviruses. These two observations gave rise to the formal possibility that Twist expression does not directly induce an EMT in HMLE cells, but rather puts a differential stress on the existing epithelial and mesenchymal cells. The resultant outgrowth of the hardier mesenchymal cells over several population doublings would be difficult to distinguish from the case where individual epithelial cells were slowly converted into mesenchymal cells by Twist. Zeb1 does not suffer from such caveats, because the appearance of mesenchymal morphology and markers in Zeb1-infected cells occurs within 1-2 days, well before mesenchymal cells can outgrow the major epithelial population. Zeb1 therefore is able to induce a true EMT by differentiation of individual epithelial cells, as opposed to an apparent EMT by selecting out existing mesenchymal cells.

Zeb1-induced EMT: transient or stable?

It was observed that over time, usually about two weeks, epithelial cells start to reappear among the Zeb1-infected mesenchymal cells, and eventually the majority of cells in culture would be epithelial (Figure 4). It was not known if this was because Zeb1 only causes a transient EMT, or if the small numbers of cells which did not undergo EMT for whatever reason were able to outgrow the now-majority mesenchymal population. Under normal culture conditions without additional stress, mesenchymal HMLE cells grow more slowly than their epithelial counterparts, so it is very possible for the latter case to be true. Even though the lentivirus had two selectable features (puromycin resistance and red-fluorescent protein), efforts to select out only the cells that had undergone EMT failed to prevent the reappearance of the epithelial cells.

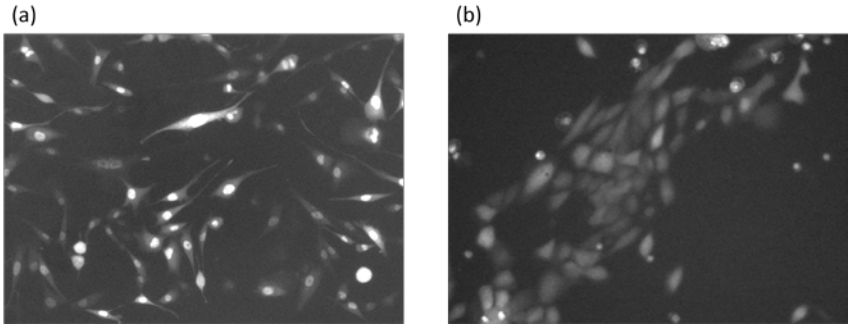

Figure 4 Reappearance of epithelial cells in HMLE cells polyclonally infected with Zeb1-expressing lentivirus. (a) 5 days post-infection, infected cells which are RFP-positive have mesenchymal morphology and some nuclear localization of RFP fluorescence. (b) Infected cells that were sorted for RFP-positivity and cultured for a further 3 weeks have epithelial morphology and no nuclear localization of fluorescence.

An inducible Zeb1 expression system was deemed desirable, to enable EMT to be induced independent of the time of infection. Attempts were made to create a tamoxifen-inducible system, by means of an estrogen-receptor fusion. This was unsuccessful, as neither a C-terminal nor N-terminal fusion of the estrogen receptor fragment gave the desired characteristics of tight control and inducibility. The ER-fusion construct had enough background activity that once the cells were infected, even before adding tamoxifen, enough Zeb1 activity would be present to induce an EMT. Further addition of tamoxifen made no qualitative difference.

Subsequently, a doxycycline-inducible Zeb1 expression lentiviral construct was created, and this was successful. In the absence of doxycycline, there is no spontaneous induction of EMT, while the addition of doxycycline resulted in a fast EMT on the same timescale as the constitutively expressed constructs. Once EMT was induced by the addition of doxycycline, however, the same phenomenon of epithelial cell reappearance occurred. Finally, single-cell clones were made of HMLE cells infected with the inducible Zeb1-expression construct. Every cell is expected to have the same number of lentiviral integrations, at the same sites on the genome. Several single-cell clone lines were tested, and all were able to undergo EMT, without any reappearance of epithelial

cells, demonstrating that the reappearance of epithelial cells was an experimental artifact that could be eliminated under the right circumstances. Zeb1 induces a stable EMT.

Zeb1 is necessary to maintain the mesenchymal state

In addition to maintaining the mesenchymal state under constant exposure to doxycycline, it was found that the new single-cell clones could maintain this state even after doxycycline was withdrawn. The exogenous Zeb1 was only necessary to initiate the EMT, and not required to maintain the mesenchymal state. However, endogenous Zeb1 was found to be upregulated in the mesenchymal cells, suggesting the exogenous Zeb1 had triggered some feedback pathway that led to the upregulation of endogenous Zeb1. This raised the question of whether the endogenous Zeb1 was keeping cells in the mesenchymal state. Lentiviruses expressing short hairpin RNA (shRNA) against Zeb1 were designed and tested on HMLE cells turned mesenchymal by transient exogenous Zeb1 expression. The most effective shRNA was able to reduce endogenous Zeb1 levels and cause the mesenchymal HMLE cells to revert to an epithelial state (Figure 5). Zeb1 is therefore both sufficient to trigger the switch into the mesenchymal state, and necessary for maintaining that state. Until now, this property has never been demonstrated for any EMT-inducing factor within a single cell type.

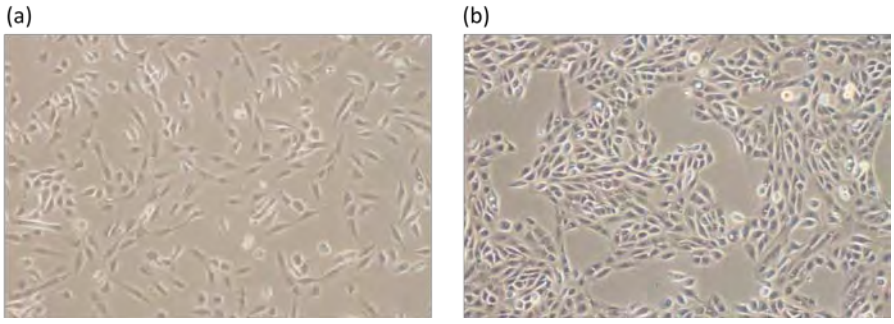

Figure 5 Mesenchymal-Epithelial Transition of HMLE cells that had previously undergone EMT. (a) HMLE single-cell clone exposed to 10 days of doxycycline followed by 2 weeks without doxycycline. (b) The same cells, but infected with Zeb1-knockdown lentivirus at the end of doxycycline treatment and cultured for 2 weeks without doxycycline. The single-cell clone stays mesenchymal without further treatment, but reverts to an epithelial morphology upon knockdown of endogenous Zeb1.

Role of Zeb1 in EMT induced by other factors

Having established that Zeb1 can trigger an EMT more rapidly than previously known factors, while being apparently upregulated during EMT by said factors, this raised the possibility that the other known factors work through Zeb1 to induce the EMT. An attempt was made to interfere with Zeb1 signaling to see if it was necessary for the EMT induced by other factors. Due to the possible confounding effects involved in the EMT observed by Twist overexpression, Snail-induced EMT was chosen as the process to be investigated.

Since the single-cell clones with inducible Zeb1 proved to be a reliable system and eliminated many of the pitfalls associated with polyclonally-infected cell populations, single-cell clones with inducible Snail were generated in a likewise manner. However, all the single-cell clones which tested positive for ability to overexpress Snail under doxycycline induction were nevertheless unable to undergo EMT. No morphological change or change in characteristic epithelial and mesenchymal markers was observed, nor was Zeb1 upregulated, despite several weeks of exposure to doxycycline. The difference between the doxycycline-inducible Snail and constitutively expressing Snail vectors stem primarily from the absolute expression levels, with the latter giving higher levels. For lack of a better alternative, the constitutively expressing Snail vector was used to induce EMT for this experiment. The main drawback of this system is the inability to turn off the exogenous Snail after EMT had been induced. The fact that Snail-induced EMT can only be accomplished with high levels of expression gives rise to the possibility that it is acting promiscuously at these levels, perhaps binding to E-boxes it would normally not bind at physiological levels.

An epithelial single-cell clone of HMLE was polyclonally infected with the Snail-expressing lentivirus, and allowed to undergo EMT. Once the mesenchymal phenotype was acquired, the cells were further infected with lentivirus expressing shRNA against Zeb1, or a control hairpin. The knockdown of Zeb1 with two independent hairpins resulted in a shift in the markers from the mesenchymal to the epithelial set (Figure 6). This result suggests that Snail-induced EMT works through Zeb1, putting Zeb1 downstream of Snail in the EMT regulatory circuit.

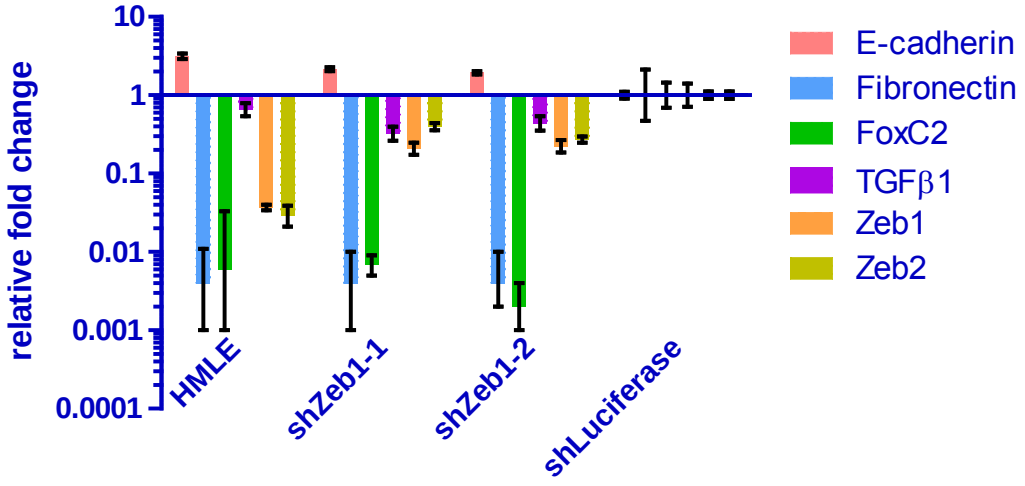

Figure 6 Realtime PCR showing partial reversion of Snail-induced EMT by Zeb1 knockdown. Knockdown of Zeb1 with two independent hairpins resulted in a concomitant increase in E-cadherin and decrease in various mesenchymal markers, moving the marker profile towards that of uninfected epithelial HMLE cells.

4. Demonstrate that key components of the EMT can profoundly influence the ability of tumorigenic cells to metastasize *in vivo*

Early attempts to test the effect of Zeb1-induced EMT *in vivo* ran into the complication of outgrowth of epithelial cells. Over the timescale of the *in vivo* experiment, epithelial cells would be expected to overwhelm the slower-growing mesenchymal cells, rendering a null result uninterpretable. A null result was in fact obtained, whereby there was no metastasis resulting either from the control cells or cells that had undergone Zeb1-induce EMT.

With the development of the single-cell clones with inducible Zeb1, a new attempt was made to address the ability of EMT to influence metastasis. The single-cell clones were polyclonally infected with retrovirus to express mutant rat Neu, which is known to transform HMLE bulk cells, enabling them to grow as xenografts in mice. Since mesenchymal cells are more anoikis-resistant, they are able to establish primary tumors in lower numbers. This was not the focus of this study, and so in order to circumvent the difference in tumor-initiating ability, the cells were injected into mice while still in the epithelial state. The aim was to induce EMT *in vivo* after the primary tumors had established, and then study the difference in metastatic ability.

The first attempt with this new experimental design was not successful, due to the unexpectedly low tumorigenicity of the injected cells. Less than 20% of the mice developed tumors despite a substantial load of 500,000 cells injected per site. A second attempt was made using a different single-cell clone, polyclonally infected with a retrovirus to express mutant Ras, another known transforming oncogene. One million cells were injected per site, but this still resulted in less than 50% tumorigenecity. As of this writing, the experiment is still ongoing, and new attempts are being made with different mouse strains.

5. Correlate activity of key components of the EMT with metastasis of human breast cancers and other cancers, using laser capture microdissected tumor samples

Not yet underway.

6. Develop a usable signature of EMT that can be used as a predictor of a primary tumor's tendency to metastasize

Not yet underway.

Key Research Accomplishments

- Generated EMT core gene signature from microarray data
- Identified Zeb1 as a key candidate transcription factor mediating the EMT
- Demonstrated Zeb1 induces the EMT more quickly than previously known factors

- Demonstrated Zeb1 induces a stable EMT
- Demonstrated exogenous Zeb1 is only transiently required for stable EMT
- Demonstrated endogenous Zeb1 is necessary for maintenance of mesenchymal state
- Demonstrated Zeb1 acts downstream of Snail in the EMT regulatory circuit

Reportable Outcomes

- EMT core gene signature
- Human Mammary Epithelial Cell lines expressing Zeb1 and relevant control lines
- Human Mammary Epithelial Cell lines expressing short hairpin RNA targeting Zeb1 and relevant control lines
- Single-cell clones of Human Mammary Epithelial Cell lines with ability to undergo Zeb1-induced stable EMT upon transient exposure to doxycycline
- Transformed derivative of above single-cell clones capable of forming tumors in mice for *in vivo* experiments

Conclusions

This study has shown that Zeb1 is a key mediator of the EMT, and suggests that it is qualitatively different from other known factors involved in the EMT. The ability to induce EMT in a short timeframe sets it apart from other factors, and its importance was demonstrated by the fact that it is both sufficient and necessary within a single cell system. There is also some evidence that it is acting downstream of Snail in the EMT regulatory circuit. Further, the ability to induce a clear EMT without possible involvement of cell-selective effects makes it a very clean system for studying the EMT process. The inducible single-cell clones hold great potential for further studying the EMT, both *in vitro* and *in vivo*, although some technical issues remain to be resolved. In particular, the ability to activate exogenous Zeb1 at will means that the role of EMT in metastasis can be separated from its role in tumor-initiation, something that has not been clearly delineated at this time. The ability to turn off exogenous Zeb1 simply by doxycycline withdrawal means that cells are no longer being forced to stay mesenchymal by artificial means. These cells are yet another useful tool for studying the factors and signaling pathways required to maintain cells in the mesenchymal state. The gene network acting downstream of Zeb1 is of great interest, and merits in-depth study.

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